**Exhibit 6**

# Intranasal Immunization with Plasmid DNA-Lipid Complexes Elicits Mucosal Immunity in the Female Genital and Rectal Tracts[1]

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The development of vaccines against pathogens transmitted across the genito-rectal mucosa that effectively stimulate both secretory IgA Abs and cytotoxic T lymphocytes in the genital tract and CTL in the draining lymph nodes (LN) has proven a major challenge. Here we report a novel, noninvasive approach of genetic vaccination via the intranasal route. Such vaccination elicits immune responses in the genital and rectal mucosa, draining LNs, and central lymphoid system. Intranasal immunization with plasmid DNA-lipid complexes encoding the model Ag firefly luciferase resulted in dissemination of the DNA and the encoded transcript throughout the respiratory and gastrointestinal tracts, draining LNs, and spleen. Complexing the plasmid DNA with the lipid DMRIE/DOPE enhanced expression of the encoded protein in the respiratory tract, increased specific secretory IgA Ab in the vaginal and rectal tracts, and increased the circulating levels of specific IgA and IgG. In addition, intranasal DNA immunization resulted in generation of Ag-specific CTL that were localized in the genital and cervical LNs and spleen. These results suggest that intranasal immunization with plasmid DNA-lipid complexes may represent a generic immunization strategy against pathogens transmitted across the genito-rectal and other mucosal surfaces. *The Journal of Immunology*, 1999, 162: 254–262.

T he mucosal surfaces of the cervix, vagina, and rectum represent the primary site for transmission of several viruses associated with global morbidity, including HIV and herpes simplex (HSV).[3] To prevent virus transmission across the mucosal epithelium, and to prevent virus dissemination to the regional lymph nodes (LNs) or target organ(s), effective vaccines should ideally stimulate immune responses at the mucosal tissues and the associated LNs. Such responses include secretory IgA (sIgA) and IgG Abs that are reported to mediate virus neutralization (1, 2) and prevent virus adhesion (2), and intraepithelial IgA, which may inhibit intracellular virus replication (3). In addition, stimulation of proliferative and cytotoxic T lymphocytes may be essential for virus clearance from mucosal tissues to prevent virus dissemination if the mucosal barrier is breached. Detection of cell-mediated cytotoxicity and Ab-secreting cells in genital tissues following virus infection (4–7) and evidence for the migration of genital LN-derived CD8^{+} T cells to the genito-rectal mucosa during infection with HSV and SIV (6, 8) suggest that an appropriate vaccine and immunization strategy may be able to elicit both secreted Abs and CTL in the genital and rectal tracts. To date, the induction of both cell-mediated and humoral immunity has proven a major challenge for the development of an effective mucosal vaccine. Systemic immunization has in general failed to elicit long

term protective immunity in the genital tract (9, 10). In contrast, mucosal (oral or nasal) administration of particulate Ag carriers, including Ty particles, biodegradable microspheres, and recombinant proteins conjugated to cholera toxin B subunit, has been effective in stimulating sIgA (2). However, with few exceptions (11–13), these mucosal vaccines do not induce MHC class I-restricted CTL, presumably due to the requirement for endogenous synthesis of proteins for processing and presentation of peptides with MHC class I molecules.

Recently, several groups have reported the ability of DNA expression vectors encoding foreign proteins to elicit both cellular and humoral immune responses to the encoded protein following immunization by the i.m. (14–18) or the intradermal (19, 20) route. Protection against systemic infection in several murine and non-human primate models by this technology (16, 18, 21, 22) suggests that this novel approach may also elicit protective mucosal immunity, provided the plasmid DNA is targeted to the specialized inductive sites of the mucosal immune system. Such sites may include the organized lymphoid tissues associated with the gut, rectum, bronchus, and nasopharynx or the draining LNs (23–25). Ags presented at these sites induce the priming and homing of IgA-committed B cells and effector T cells to a variety of mucosal tissues (25, 26). Such an approach has the advantage of potentially achieving both systemic immunity and secretion of Ag-specific IgA at distant mucosal sites.

The nasal mucosa presents an attractive, noninvasive route for the delivery of DNA vaccines. Based on the concept of a common mucosal immune system (26, 27), we have addressed whether genetic immunization by the intranasal route will elicit both systemic and secretory Ab responses at the cervical-vaginal and rectal mucosa and MHC-restricted CTL in the regional LNs and spleen. To evaluate the feasibility of this approach for the development of vaccines against sexually transmitted pathogens, we used firefly luciferase (*Photinus pyralis*) as a model Ag to investigate 1) the efficiency of cationic lipids to augment gene expression and indirectly influence Ag presentation at the nasal mucosa, 2) the distribution and duration of plasmid DNA

and mRNA following vaccination, and 3) the efficiency of cationic lipid-mediated genetic immunization to induce systemic and mucosal immunity in the female genital tract.

## Materials and Methods

### Plasmid DNA construction and purification

Firefly luciferase cDNA was subcloned from plasmid pGEM-luc (Promega, Madison, WI) into the *BgI*II site of the eukaryotic expression vector VIJ (28). This vector consists of a pUC-19 backbone with the CMV IEI enhancer, promoter and intron A transcription regulatory elements, a bovine growth hormone polyadenylation sequence, and an ampicillin resistance gene (28). Plasmid VIJ-luc was transformed into *Escherichia coli* DH5α and purified by alkaline lysis and double cesium chloride gradient ultracentrifugation (29) followed by ethanol precipitation. Plasmid DNA was resuspended in pyrogen-free water. The spectrophotometric $A_{260}/A_{280}$ ratios were between 1.7 and 2.0. The endotoxin content was <5 ng/mg of plasmid DNA determined by the *Limulus* amebocyte lysate assay (Sigma, St. Louis, MO).

### Plasmid DNA-lipid formulation

To enhance the efficiency of plasmid DNA uptake by nasal epithelium, plasmid DNA was complexed with DMRIE/DOPE ((+)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide and 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine) provided by our colleagues at Vical (San Diego, CA). Characterization of this lipid has been reported previously (30). The lipid formulation was prepared by dissolving DMRIE (30) and DOPE into chloroform and was lyophilized by rotary evaporation and overnight evacuation (31). All plasmid DNAs were complexed by incorporating the DNA into the lipid using a 1/1 molar ratio of DMRIE/DOPE at a DNA/lipid ratio of 5/1 (w/w) based on the mass of DMRIE in the liposome complex. The final plasmid DNA concentration was 2.5 mg/ml.

### Immunization

Seven- to eight-week-old female BALB/c mice (Harlan Olac, Bichester, U.K.) received 20 µl of plasmid DNA-lipid complexes containing 50 µg of plasmid DNA to the nares under general anesthesia (Hypnovel, Roche, Welwyn Garden City, U.K.; and Hypnorm, Janssen, Beerse, Belgium). In certain experiments, the quadriceps muscles of anesthetized mice were injected with 50 µg of DNA in saline. Animal care throughout the study was in accordance with U.K. Home Office guidelines.

### Measurement of luciferase expression

At varying times following intranasal immunization, animals were euthanized with an overdose of anesthesia (Hypnovel and Hypnorm). Nasal tissue was excised, weighed, and snap-frozen on dry ice. Frozen tissues were homogenized in 100 µl of lysis buffer (Promega, Cell Culture Lysis Reagent) and centrifuged for 3 min at 10,000 × g, and 20 µl of the supernatant was assayed for luciferase enzyme activity by chemiluminescence assay (Promega Luciferase Assay System) using a Turner TD-20e luminometer. The luciferase content of samples was calculated from relative light units using a standard curve of purified firefly luciferase (Sigma). The total protein concentration of each nasal extract was determined by the Bio-Rad DC protein assay (Richmond, CA). Luciferase values were expressed as picograms per milligram of total protein extract.

### In situ hybridization

In situ hybridization was performed on snap-frozen unfixed nasal tissue as described previously (32). A fluorescein-labeled antisense luciferase RNA was synthesized in vitro by run-off transcription from plasmid pGEM-luc DNA (Promega), incorporating fluorescein-12-UTP. Frozen sections were dehydrated in alcohol, and 30 ng of the antisense probe in hybridization buffer was added to each slide (hybridization buffer is 1× Denhardt's solution, 4× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 100 µg/ml polyadenylitic acid, 10% dextran sulfate, 50% deionized formamide, and 100 µg/ml denatured herring sperm DNA). Slides were incubated overnight at 55°C and washed for 30 min at room temperature with 0.1× SSC followed by 30 min at 65°C with 0.5× SSC, for 10 min at room temperature with 0.5× SSC, and for 5 min at room temperature with 0.1 M Tris (pH 7.5) containing 0.4 M NaCl. Sections were incubated for 30 min at room temperature with 0.5% Amersham blocking agent (RPN 3023, Amersham, Aylesbury, U.K.) in 0.1 M Tris (pH 7.5) containing 0.4 M NaCl, followed by overnight incubation with an alkaline phosphatase-labeled anti-fluorescein Ab (Amersham). Hybridization signal was detected

by incubation with 5-bromo-4-chloride-indolyl-phosphate and 4-nitro blue tetrazolium chloride (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

### PCR

Individual tissues isolated with separate sterile instruments (as a precaution to prevent cross-contamination) were snap-frozen and stored at −70°C. DNA was extracted from minced tissues in 1.2 ml of lysis buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8), 0.5% SDS, and 0.1 mg of proteinase K/100 mg tissue) using standard techniques (28). PCR was performed in a 50-µl reaction volume containing 0.1 µg of DNA, 10 mM Tris-HCl (pH 8.3) 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3), 2 mM dNTPs, 0.75 ng of each primer, and 2 U of Taq DNA polymerase (Boehringer Mannheim). The oligonucleotide primers used were: luciferase, 5'-TCTGACGCAGGCAGTTCTATG-3' (sense) and 5'-ACTACAAGTG GAGCTATACAC-3' (antisense) amplifying a 520-bp fragment; and glyceraldehyde-3-phosphate dehydrogenase, 5'-TCATGACCACAGTC CATGCCATCAC-3' (sense) and 5'-GACCATACTGTTACTTATGC CGATG-3' (antisense) amplifying a 436-bp fragment. The samples were processed on a Biometra Trio-Thermoblock thermal cycler (Tampa, FL) programmed for one cycle at 96°C for 2 min and 40 cycles each of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min. Ten-microliter aliquots of the PCR products were analyzed on a 1.5% agarose gel and visualized with ethidium bromide.

### RT-PCR

Total RNA was extracted from frozen minced tissues using 1.0 ml of a guanidinium isothiocyanate/phenol-based monophasic extraction solution (TRIzol, Life Technologies, Grand Island, NY) as lysis buffer. After incubation for 5 min at room temperature, 0.2 ml of chloroform was added, and samples were vortexed and incubated 3 min at room temperature. The aqueous phase, separated by centrifugation for 15 min at 12,000 × g, was transferred to a new tube and combined with 0.5 ml of isopropyl alcohol. RNA was recovered by centrifugation (15 min at 12,000 × g), and following washing in 75% ethanol, the pellet was resuspended in diethylpyrocarbonate-treated water. To remove any possible contaminating plasmid DNA, RNA samples were incubated with 2.5 U of RNase-free DNase (60031, Stratagene, La Jolla, CA) for 1.5 h at 37°C, followed by phenol/chloroform extraction and ethanol precipitation. Five micrograms of total RNA was reverse transcribed using the Stratagene RT-PCR kit. As a control, reverse transcriptase was omitted from the reaction with a second aliquot of RNA. A 5-µl aliquot of each preparation (with or without reverse transcriptase) was then amplified using the oligonucleotide primers and PCR protocols described above and was analyzed by agarose gel electrophoresis.

### Collection of fluids

Serum was obtained from tail vein blood or from blood collected from the subclavian veins when mice were euthanized. Vaginal and rectal fluids were obtained by introducing 50 µl of PBS with a pipette and withdrawing it five times. Vaginal and rectal fluids were collected twice over 72 h and pooled from individual mice. The fluids were stored at −20°C and centrifuged before assay.

### ELISA for luciferase Ab

Firefly luciferase (Sigma) in 0.1 M carbonate-bicarbonate buffer, pH 9.6, was absorbed onto microtiter plates (Immulon 4, Dynatech, Chantilly, VA) overnight at 4°C. Plates were washed twice with PBS containing 0.05% Tween-20 (Merck Chemicals, Poole, U.K.) and blocked with 3% BSA (Sigma) for 2 h at room temperature. Plates were washed and incubated with 50 µl of serial dilutions of serum or vaginal or rectal fluids (in duplicate) for 2 h at room temperature. Plates were then washed and incubated for 2 h at room temperature with 100 µl of affinity-purified goat anti-mouse IgA or IgG conjugated to alkaline phosphatase (Sigma). Ab binding was detected by addition of p-nitrophenyl-phosphate disodium substrate (Sigma) and was measured by the OD emitted at 405 nm on a Bio-Rad model 2550 ELISA plate reader. The results were expressed as the lowest dilution giving an OD of 3 SD above the mean of the background sample for all serum samples and rectal and vaginal washings.

### Measurement of sIgA

To detect the secretory component associated with luciferase-specific IgA, vaginal fluids were pooled separately (five per group) from mice immunized with plasmid DNA-cytofectin complexes containing either VIJ-Luc or the empty VIJ vector. Fluids were clarified by centrifugation at

10,000 × g and concentrated 10-fold with Centricon-30 microconcentration filters (Amicon, Beverley, MA). Serial dilutions of vaginal fluids were incubated with luciferase Ag-coated ELISA plates (as described above). Plates were then washed and incubated for 2 h at room temperature with 100 μl of a goat antiserum to rat secretory component that is cross-reactive with mouse secretory component (33). This antiserum has been repeatedly absorbed with rat serum and shows no binding to rat or mouse IgA (33). After washing, plates were incubated with 100 μl of affinity-purified sheep anti-goat IgG conjugated to alkaline phosphatase (Sigma), and specific binding was detected by addition of *p*-nitrophenyl-phosphate disodium substrate as indicated above.

*Measurement of CTL activity*

Mononuclear cells isolated from the spleen or cervical or iliac LNs were resuspended in RPMI supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Sigma), and $5 \times 10^{-5}$ M 2-ME (Eastman Kodak, Rochester NY). Responder cells ($4 \times 10^7$) were cocultured with $4 \times 10^6$ syngeneic UV and gamma-irradiated (3000 rad) spleen cells infected 16 h previously with a recombinant Adeno 5 virus expressing luciferase (Ad5-Luc 3) (34). Effector cells were harvested 5 days following in vitro culture and washed. Serial dilutions of effector cells were tested in a standard $^{51}$Cr release assay (35) with $5 \times 10^3$ $^{51}$Cr-labeled target cells. These included P815, a H-$2^d$ cell line either uninfected or infected 16 h previously with 5 plaque-forming units/cell vaccinia virus expressing luciferase (VVLuc) (36) or vaccinia virus expressing SIV gag p55 (VVgag; provided by M. Mackett, Manchester, U.K.) and RMA, a H-$2^b$ target cell line infected with 5 plaque-forming units/cell VV Luc. Cytotoxic activity was calculated as the percent specific lysis from the formula (experimental release − spontaneous release)/(total release − spontaneous release) × 100. Total release was determined by incubation of targets with 5% Triton X-100 containing 2% SDS.

*Statistics*

To determine the significance of differences in the concentration of luciferase expressed in nasal extracts following immunization with naked plasmid DNA and plasmid DNA-cytofectin complexes, an analysis of variance was used. For all other statistical analyses, Student's *t* test was used. A *p* value of 0.05 or less was considered significant in both tests.

# Results

*Expression and duration of luciferase in nasal epithelium*

The nasal epithelium and associated secretions present a formidable physical and chemical barrier to substances entering the respiratory tract (37). Such barriers may preclude the efficacy of nasal immunization with nucleic acid. Therefore, we first addressed the efficiency of DNA uptake and protein expression by intranasal immunization. In view of the reported inefficiency of naked DNA uptake, particularly by tissues other than cardiac and skeletal muscle (38) or skin, we evaluated protein expression following cytofectin-mediated plasmid DNA delivery using DMRIE/DOPE compared with naked DNA alone. Plasmid DNA-cytofectin complexes containing the firefly luciferase expression vector VIJ-Luc or the empty plasmid V1J were administered topically to the nares of BALB/c mice. Three days following instillation, extracts of nasal epithelium isolated from recipients of V1J-Luc cytofectin complexes showed a 30-fold increase in luciferase protein compared with recipients of naked plasmid DNA (Fig. 1*A*; *p* < 0.001). Luciferase expression persisted for at least 28 days following transfection with plasmid DNA-cytofectin complexes containing V1J-Luc. Although the mean concentration of luciferase declined by day 7 to 33% of the day 3 value (found to be the peak, relative to day 1 or 2; L. S. Klavinskis et al., unpublished observations), expression was maintained at a residual level corresponding to 4.5% of the day 3 value on day 28 (Fig. 1*A*). An analysis of variance of the concentration of luciferase expressed in nasal tissue at each time point indicated a significant increase in expression by administering plasmid DNA complexed with DMRIE/DOPE compared with naked DNA alone ($F(1,24) = 124.59$; *p* < 0.001).

In situ hybridization of nasal epithelium (from mice immunized with V1J-Luc cytofectin complexes) with an antisense luciferase

RNA probe showed hybridization signal predominantly in the squamous epithelium, with occasional cells in the lamina propria showing hybridization (Fig. 1*B*). No signal was detected in sequential tissue sections treated with RNase and hybridized with the luciferase probe, demonstrating that the hybridization signal was specific to the RNA transcripts (Fig. 1*C*). Similarly, no hybridization signal was detected from mice transfected with plasmid DNA-cytofectin complexes containing the empty plasmid, V1J, demonstrating specificity to luciferase (Fig. 1*D*).

*Distribution and duration of plasmid DNA and mRNA*

Since nasal instillation with a DNA vaccine could potentially target and prime the organized mucosal associated lymphoid tissues of the upper and lower respiratory tract, we used a PCR-based approach to determine the location of plasmid DNA and luciferase mRNA following intranasal administration. Using luciferase-specific primers, amplified Luc DNA PCR product was detected in isolated tissues throughout the respiratory tract (nasopharynx, trachea, and lung) and also the gut, spleen, and cervical and mesenteric LNs (Fig. 2*a*) at 3 days following intranasal inoculation. The absence of PCR product from the brain, liver, quadriceps muscle, and ovary (Fig. 2*a*) suggested that the distribution of plasmid DNA was not random, but was restricted to the respiratory and gastrointestinal tract as well as the spleen. The strongest PCR signal was consistently observed for nasal tissue, lung, and cervical LNs. RT-PCR analysis of RNA isolated on day 3 demonstrated a pattern of luciferase mRNA tissue expression that was the same as that seen for plasmid DNA (Fig. 2*b*). Southern blot analysis of the RT-PCR products confirmed that the PCR product was the luciferase sequence (not shown). No signal was obtained from tissues isolated from animals administered plasmid DNA-cytofectin complexes containing the empty vector DNA (Fig. 2*c*). Thus, intranasal immunization is associated with DNA delivery to mucosal surfaces throughout the respiratory and gastrointestinal tract. Preliminary data from PCR studies indicate that intranasal immunization is associated with transfection of dendritic cells in the organized lymphoid tissue of the nasal cavity and the draining LNs (C. Barnfield and L. S. Klavinskis, unpublished observations).

A potential concern with this immunization strategy, unlike vaccination with DNA by the i.m. route, is that persistence of the encoded gene product in mucosal tissues may have the potential to induce systemic tolerance. We therefore investigated the duration of luciferase transcripts following intranasal immunization. RT-PCR analysis of tissues from each of three animals demonstrated the presence of luciferase transcripts in nasal tissue for at least 28 days and in the lung and cervical LNs for at least 14 days. In the trachea, gut, and mesenteric LNs, variable expression was detected for at least 7 days. In the spleen, transcripts were consistently detected only on day 3 (Table I).

*Humoral responses induced by intranasal DNA immunization*

We next investigated whether intranasal immunization with either naked plasmid DNA or plasmid DNA complexed with DMRIE/DOPE would elicit luciferase-specific IgA and IgG Abs in serum and vaginal and rectal fluids. Four weeks after the primary immunization, mice immunized with the naked plasmid V1J-Luc showed negligible serum anti-luciferase IgG or IgA Ab by ELISA; the mean titers were 72 and 24 above the pre-immunization value (Fig. 3, *a* and *b*). In contrast, all 10 mice immunized with V1J-Luc cytofectin complexes demonstrated serum anti-luciferase IgG and IgA Ab, with mean titers of 3061 and 576, respectively (Fig. 3, *a* and *b*). Analysis of the Ag-specific serum IgG isotype from these mice indicated a 1.6-fold increase in IgG1 Ab relative to IgG2a, which may represent a
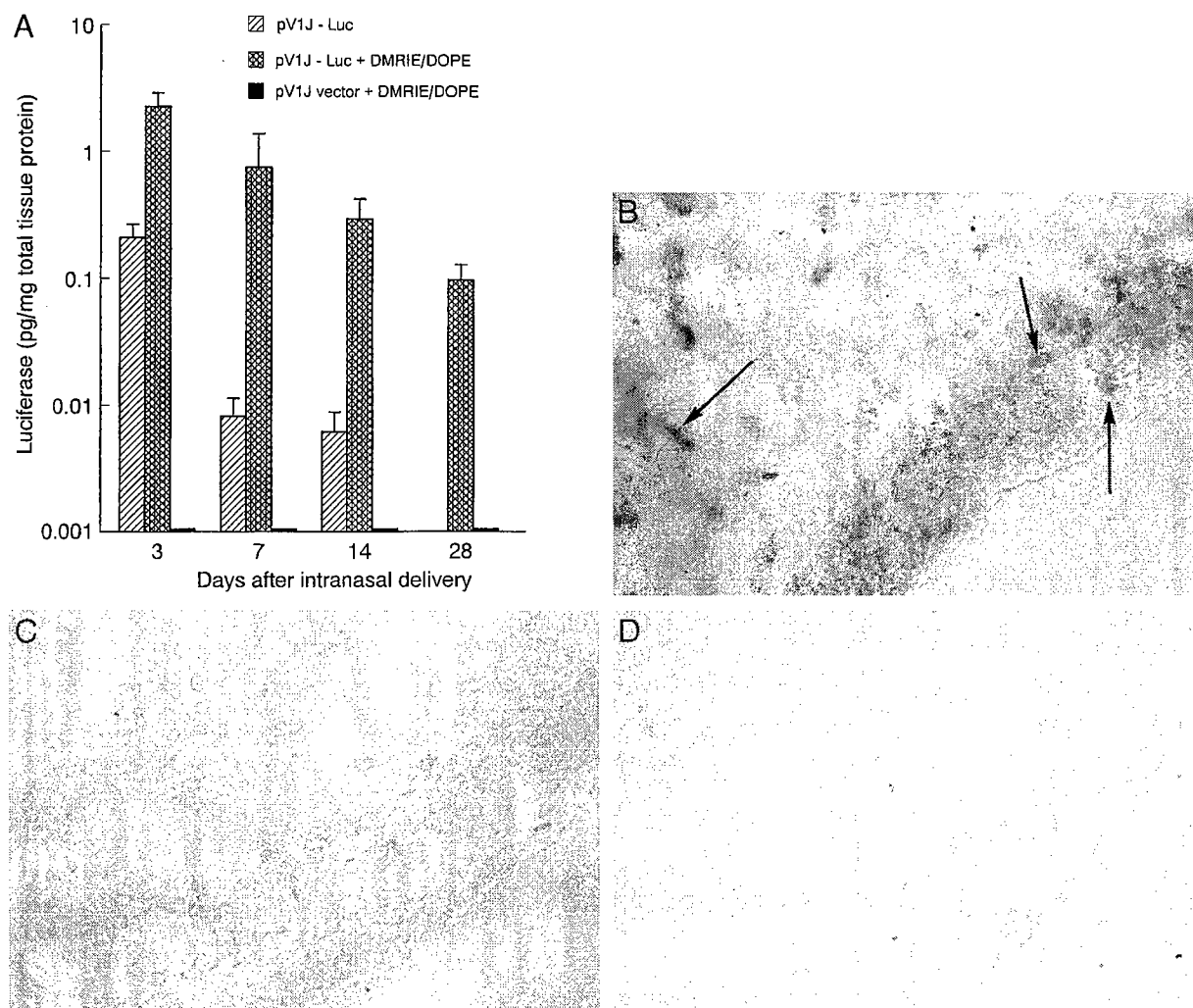
**FIGURE 1.** Detection and duration of luciferase expression in nasal tissue from BALB/c mice which received 50 μg of either plasmid pV1J-Luc or the empty vector V1J. *A*, Duration of luciferase protein expression in mice receiving the naked plasmid pV1J-Luc, luciferase DNA-lipid complexes (V1J-Luc and DMRIE/DOPE), or DNA-lipid complexes containing the empty vector (pV1J and DMRIE/DOPE). Nasal extracts were prepared in lysis buffer and analyzed for luciferase activity by chemiluminescence assay as described in *Materials and Methods*. Luminescence (light units) was converted to picograms of luciferase from a standard curve established from luminescence produced by purified luciferase. Data are expressed as the mean ± SEM ($n = 5$) in terms of luciferase concentration (picograms per milligram of total protein). Luciferase expression was significantly enhanced in mice receiving luciferase DNA-lipid complexes compared with recipients of luciferase DNA alone by analysis of variance ($F(1,24) = 124.59$; $p < 0.001$). *B–D*, Detection of luciferase RNA in nasal epithelium by in situ hybridization 3 days following administration of plasmid DNA-lipid; all sections were hybridized with a luciferase antisense RNA probe. *B*, Representative section of nasal epithelium after administration of pV1J-Luc; *C*, an adjacent section pretreated with RNase; *D*, a representative section from epithelium transfected with the empty vector V1J.

mixed Th1/Th2 response (data not shown). To determine whether intranasal immunization with plasmid DNA also stimulated a mucosal IgA response in the female genital tract and rectum, we analyzed rectal and vaginal fluids for luciferase Ab. An Ag-specific IgA response in rectal and vaginal fluids could only be detected in plasmid DNA-lipid-immunized mice (Fig. 3, *c* and *d*). A second immunization via the i.m. route with the naked V1J-Luc plasmid still failed to elicit detectable luciferase-specific IgA Ab in rectal or vaginal fluids (data not shown). Thus, cytofectins were required for successful immunization with DNA by the intranasal route.

*Correlation between immunization route and secretory IgA production in vaginal and rectal fluids*

Detection of luciferase IgA in vaginal or rectal fluids could infer transudation from serum, perhaps through trauma or abra-

sion through sampling. To exclude this possibility, we addressed whether systemic immunization with naked V1J-Luc in saline into the quadriceps muscle would elicit a detectable luciferase IgA response in vaginal fluids. As indicated in Fig. 4*a*, i.m. delivery of plasmid DNA-lipid complexes did not induce luciferase-specific IgA in vaginal fluid, although this route induced a high titered IgG response in serum. For example, at 8 wk postimmunization, the reciprocal Ab titer was 2176 (Fig. 4*a*). The ratio of luciferase IgG to IgA Ab titers in the vaginal fluids of intranasal immunized mice was significantly lower than that in serum (0.10 and 5.3, respectively; $p < 0.05$, by paired Student's *t* test). To further investigate whether luciferase IgA detected in vaginal fluid was mainly of the secretory type, we analyzed pooled vaginal fluids by ELISA using luciferase as the target Ag and antisera to the secretory component as the detection component of the assay. The anti-secretory component antisera used in these assays does not bind murine

serum IgA (33). Vaginal fluid from mice immunized with plasmid DNA-cytofectin complexes containing VIJ-Luc, but not the empty vector, demonstrated that significant titers of luciferase IgA Ab associated with secretory component were induced (Fig. 4b). These results clearly support the conclusion that cytofectin-mediated intranasal immunization elicits an Ag-specific vaginal sIgA response.

### CTL induction in genital lymph nodes and spleen

An ideal vaccine capable of preventing transmission or dissemination of sexually acquired pathogens may rely on the ability of the vaccine to elicit Ag-specific CTL in genital/rectal tissues and draining lymph nodes. We therefore examined whether intranasal immunization with plasmid DNA-cytofectin complexes would elicit CTL in the spleen and iliac lymph nodes draining the genito-rectal mucosa. Splenocytes from H-2$^d$ mice immunized intranasally with VIJ-Luc DMRIE/DOPE complexes demonstrated an MHC-restricted luciferase-specific CTL response following in vitro expansion (Fig. 5a). This response was specific to immunization with DNA encoding luciferase, since mice immunized with cytofectin complexes containing only the empty VIJ vector failed to show specific recognition and lysis of luciferase-expressing target cells (Fig. 5b). Immunization with DNA cytofectin complexes encoding luciferase elicited a stronger CTL response in the spleen than immunization with naked DNA encoding luciferase (62 and 21% specific lysis, respectively, at an E:T cell ratio of 50:1; data not shown). Mononuclear cells isolated from the cervical (Fig. 5c) and iliac (Fig. 5d) LNs elicited a vigorous MHC-restricted luciferase-specific CTL response. Thus, intranasal immunization with plasmid DNA-lipid complexes induces CTL in LNs draining the nasopharynx and iliac LNs draining the genito-rectal mucosa.

## Discussion

There is increasing evidence to suggest that protection of the female genital reproductive tract against infection by sexually transmitted pathogens is facilitated by the development of a sustained IgA response in genital secretions (39) and by Ag-specific CTL in the epithelium and submucosa of the cervix and vagina (4, 5, 40). Induction of both cellular and humoral immune responses in the genital tract has generally been associated with active genital infection (4, 5) or with vaccination with attenuated organisms or replicating vaccine vectors (6, 10, 40, 41, 42). Here we report that

a noninfectious genetic vaccine administered to the nasal mucosa elicits Ag-specific sIgA in vaginal fluids and MHC-restricted CTL in genital LNs.

Our results show that the delivery vehicle and route of immunization are critical factors governing the efficiency with which plasmid DNA can elicit specific vaginal and rectal sIgA. The absence of detectable Ag-specific IgA in vaginal and rectal fluids after i.m. immunization with plasmid DNA (Fig. 4) is consistent with a preferential requirement to target mucosa-associated lymphoid tissues to initiate immune responses in the vagina. Intranasal immunization with a plasmid DNA-lipid complex was shown to elicit vaginal IgA Ab that was predominantly of the secretory form, as indicated by the presence of secretory component associated with specific IgA (Fig. 4b). Interestingly, direct immunization of the vagina with naked plasmid DNA does not appear to be particularly effective at inducing sIgA in the genital tract (43), consistent with the results of studies using recombinant Ags (44, 45). This difference in the efficiency of priming for genital responses by plasmid DNA may reflect the lack of Ag-sampling M cells and functional mucosa-associated lymphoid tissue able to initiate immune responses in the vagina (46). The barrier function of the cervico-vaginal epithelium at estrus (46, 47) and the requirement for delivery vehicles such as DMRIE/DOPE to penetrate the mucosal epithelium and enhance gene expression may also play a role.

Complexing plasmid DNA with cytofectins for nasal administration was found to be essential to elicit a specific vaginal IgA response. The cytofectin also increased the specific serum IgA and IgG Ab titers (Fig. 3a). We propose that these effects may be due to an increase in Ag presentation of the DNA-encoded protein within the mucosal associated lymphoid tissue of the respiratory tract and the draining LNs. In support of this, we found that complexing plasmid DNA with DMRIE/DOPE increased the expression of the encoded protein by 30-fold (Fig. 1) in nasal tissue compared with naked DNA, consistent with previous reports of the delivery of plasmid DNA-encoded protein to the lung (48–50). The level of gene expression achieved at mucosal inductive sites may account in part for the magnitude of the sIgA and serum Ab responses achieved. This would explain the absence of specific sIgA observed by Ban and colleagues following nasal delivery of plasmid DNA complexed with DOTAP/DOPE (51), which exhibits lower transfection activity in the respiratory tract (48).

Table I.  *Tissue distribution and duration of luciferase plasmid DNA and mRNA following intranasal immunization with luciferase DNA liposomes*[a]

| Tissue | Plasmid DNA | | | | mRNA | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 | 7 | 14 | 28 | 3 | 7 | 14 | 28 |
| Nasal | + | + | + | +/− | + | + | + | +/− |
| Trachia | + | + | +/− | − | + | + | − | − |
| Lung | + | + | + | − | + | + | + | − |
| Gut | + | + | +/− | − | + | +/− | − | − |
| Cervical LN | + | + | + | − | + | + | +/− | − |
| Mesenteric LN | + | + | +/− | − | + | + | − | − |
| Spleen | + | − | − | − | + | − | − | − |
| Blood | − | NT | NT | NT | − | NT | NT | NT |
| Liver | − | − | − | − | − | − | − | − |
| Muscle | − | − | − | − | − | − | − | − |
| Brain | − | − | − | − | − | − | − | − |
| Ovary | − | − | − | − | − | − | − | − |

[a] Detection of 520-bp luciferase product by PCR from tissues isolated at indicated time point following intranasal administration of 50 μg pVIJ-Luc + DMRIE/DOPE. Data are expressed as follows: +, >100 pg PCR product detected in 3/3 mice; +/−, >1 to ≤10 pg PCR product detected in 3/3 mice; −, ≤ 0.1 pg PCR product detected in 3/3 mice. Sensitivity of detection is 0.1 pg DNA. NT = not tested.
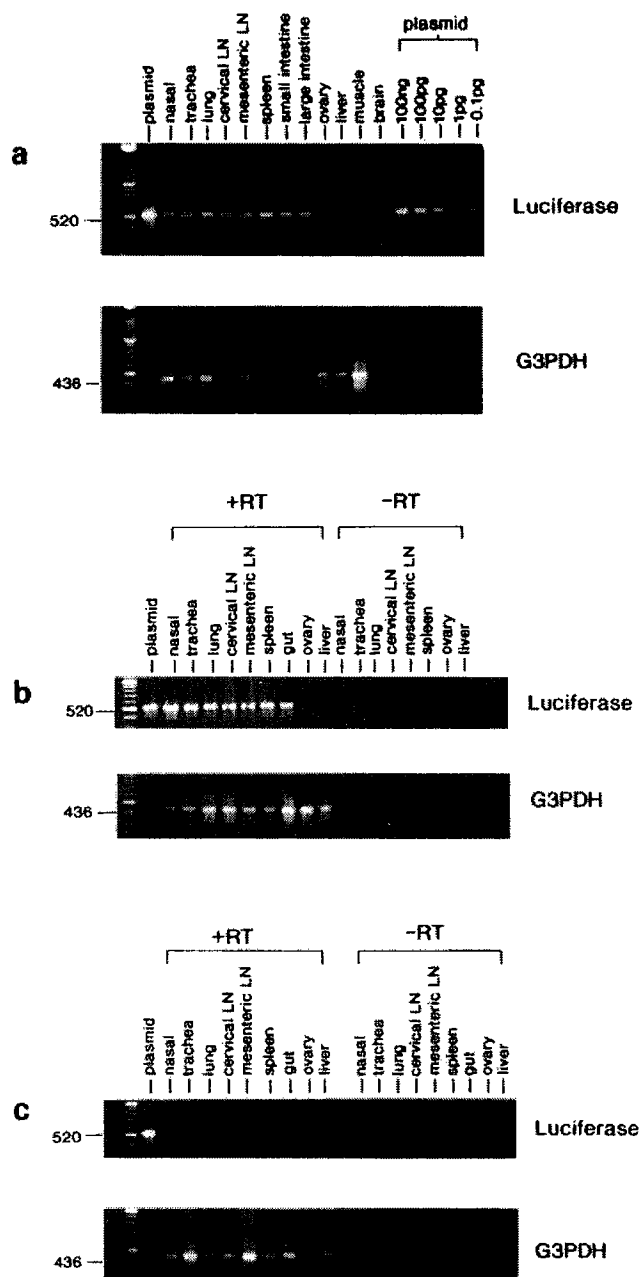
FIGURE 2. Distribution of plasmid V1J-Luc and expression of luciferase mRNA in tissues of BALB/c mice 3 days following intranasal immunization with 50 μg of pV1J-Luc cytofectin complexes. *a*, Detection of pV1J-Luc DNA. Total DNA (0.1 μg) isolated independently from each of the indicated tissues was amplified by PCR using primers specific for luciferase. Purified pV1J-Luc plasmid corresponding to 10 ng, 100 pg, 1 pg, and 0.1 pg DNA was amplified in parallel as a control. The size of the expected luciferase PCR product was 520 bp. To control for DNA content and the efficiency of the PCR, each DNA sample was also amplified with primers specific for the murine glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH). All PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. *b*, Detection of luciferase mRNA. Total RNA isolated from each tissue was treated with DNase and subjected to RT-PCR amplification with luciferase or G3PDH-specific primers (+RT). No products were seen when total RNA in the absence of reverse transcription was subject to PCR amplification (−RT). *c*, RT-PCR amplification products (luciferase/G3PDH) of total RNA isolated from animals following administration of plasmid DNA-cytofectin complexes containing only the control vector, V1J. Gut tissue in *b* and *c* represents a pool of large and small intestines.
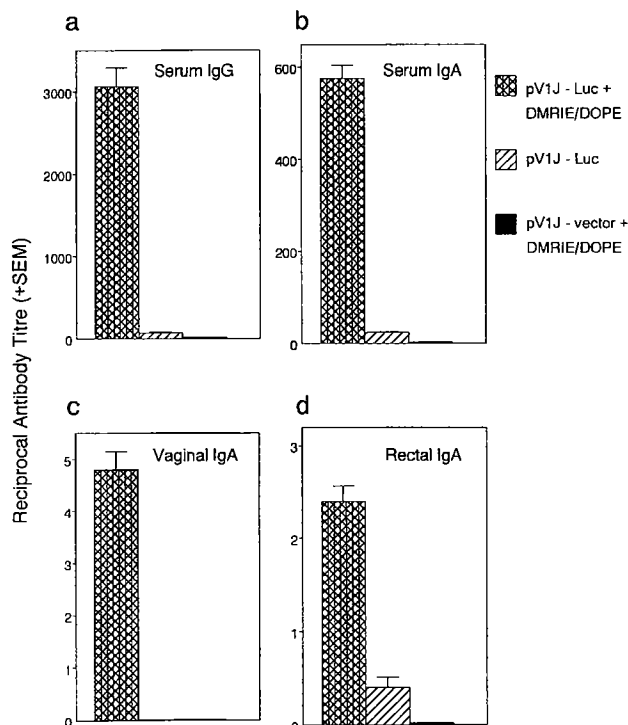


FIGURE 3. Comparison of luciferase-specific Ab induced in mice by a single intranasal immunization with luciferase DNA-lipid complexes (cross-hatched bars), empty vector DNA-lipid complexes (closed, solid bars), or luciferase DNA alone (hatched bars). Luciferase-specific Ab levels were determined 4 wk postimmunization by ELISA assay using purified firefly luciferase as the target Ag: *a*, luciferase IgG in serum; *b*, luciferase IgA in serum; *c*, luciferase IgA in vaginal fluid; and *d*, luciferase IgA in rectal fluid. The reciprocal Ab titer represents the mean ± SEM ($n$ = 10 mice). No significant binding was detected in serum or vaginal or rectal fluids obtained from mice immunized with the empty V1J vector plasmid DNA-cytofectin complexes.

Cytofectins may also provide a secondary role as an adjuvant, facilitating uptake of plasmid DNA by APCs or creating inflammation. Conventional liposomes based on phospholipids and cholesterol are reported to accumulate in the Peyer's patches following oral administration (52) and have been shown to induce elevated cellular and humoral immune responses to protein Ags (53) and plasmid DNA-encoded proteins (54, 55). Other adjuvants, including cholera toxin, have been used to promote mucosal immunity (2). However, we found no difference in gene expression in nasal tissue, serum Ab, or vaginal IgA Ab titer following coadministration of cytofectin-DNA complexes with cholera toxin, suggesting the absence of any synergistic effect (unpublished observations). In contrast, an adjuvant effect of cholera toxin in eliciting mucosal immunity with naked DNA has been reported (56). The basis for this difference requires further investigation.

It is currently thought that vaccines effective against sexually transmitted diseases may need to elicit a series of immunological barriers to limit dissemination of pathogens from the mucosal epithelium (57). Although a protective role for vaginal IgA in preventing HIV infection has been highlighted from a cohort of exposed seronegative individuals (39), and neutralization of HIV has been achieved with secretory anti-HIV Ab (1), development of successful vaccines against intracellular pathogens such as HIV, HSV, and chlamydia trachomatis may also need to elicit CTL or delayed-type hypersensitivity responses in the genital tissues and draining LNs. Our results indicate that intranasal immunization
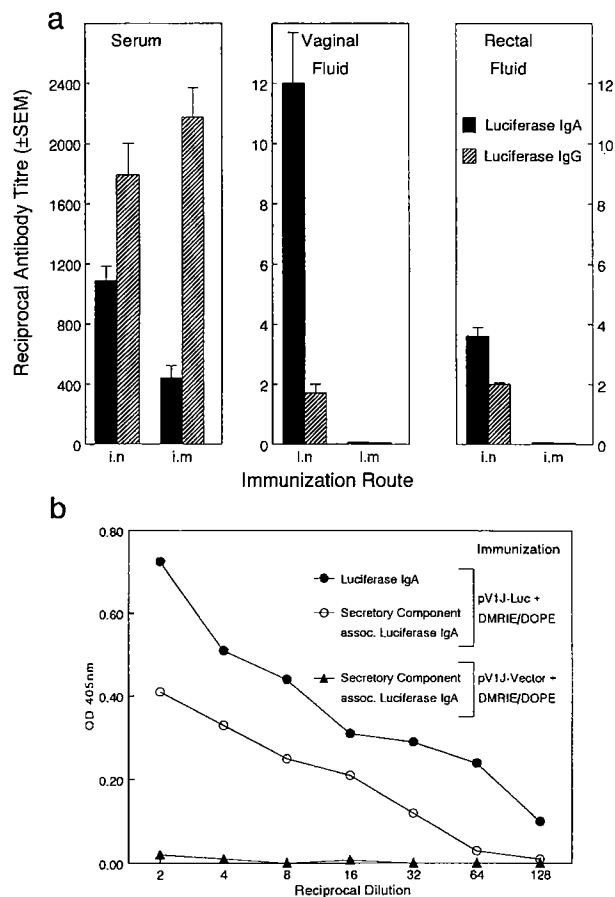
**FIGURE 4.** An sIgA response against luciferase is induced by intranasal immunization. *a*, Luciferase-specific IgA and IgG responses were analyzed in serum and vaginal and rectal fluids 8 wk following immunization with V1J-Luc and DMRIE/DOPE by the intranasal route or with V1J-Luc in saline by the i.m. route (saline is the standard methodology used in the literature for i.m. injection (1–15)). The results are expressed as the reciprocal geometric mean titer ± SEM (*n* = 10). *b*, Detection of sIgA Abs against luciferase in vaginal fluid by ELISA using purified luciferase as the target Ag. Vaginal fluids from five mice in each group were pooled and concentrated 10-fold. The binding of total vaginal IgA to luciferase Ag (●) and that of the secretory component associated with luciferase IgA binding to luciferase Ag (○) are shown for mice immunized with plasmid DNA-lipid containing V1J-Luc or the empty V1J vector only (▲) as described in *Materials and Methods*.



**FIGURE 5.** Detection of luciferase-specific CTL in mice 14 days following a second intranasal immunization with 50 μg of plasmid DNA-cytofectin complexes containing either plasmid V1J-Luc (*a*, *c*, and *d*) or the empty vector V1J (*b*). Spleen cells from a pool of three mice (*a* and *b*), cervical LN cells (*c*), and iliac LN cells (*d*) from a pool of ten mice, respectively, were restimulated with irradiated syngeneic P815 cells infected with a recombinant adenovirus (Ad5-Luc3) for 5 days, and CTL was assayed against P815 (H-2$^d$) targets infected with recombinant vaccinia virus expressing luciferase (VV Luc) or SIVgag (VVgag p55) and against RMA (H-2$^b$) targets infected with VV Luc.

with plasmid DNA-lipid complexes stimulates the induction of MHC class I-restricted CTL in the spleen and cervical LNs draining the respiratory tract (Fig. 5, *a* and *c*). Moreover, this is the first report to demonstrate that intranasal administered plasmid DNA can prime for mucosal CTL that recirculate and localize in the iliac LNs (Fig. 5*d*). In this murine system we are unable to address whether the elicited CTL also home to the vaginal mucosa. Previous studies suggest that primed CD8$^+$ T cells from the iliac LNs migrate to the vaginal mucosa following local Ag stimulation (8) and that virus-specific CTL localizing in the genital LNs are effective in clearance of virus in the vagina (6). Thus, cytofectin-mediated intranasal immunization with plasmid DNA may provide a safe, noninvasive and noninfectious, generic strategy to elicit mucosal T cells contributing an effective barrier to dissemination of pathogens from the vaginal epithelium and submucosa.

An important issue that still needs to be resolved is the underlying mechanism by which intranasal delivery of plasmid DNA elicits both systemic and vaginal immune responses. Following
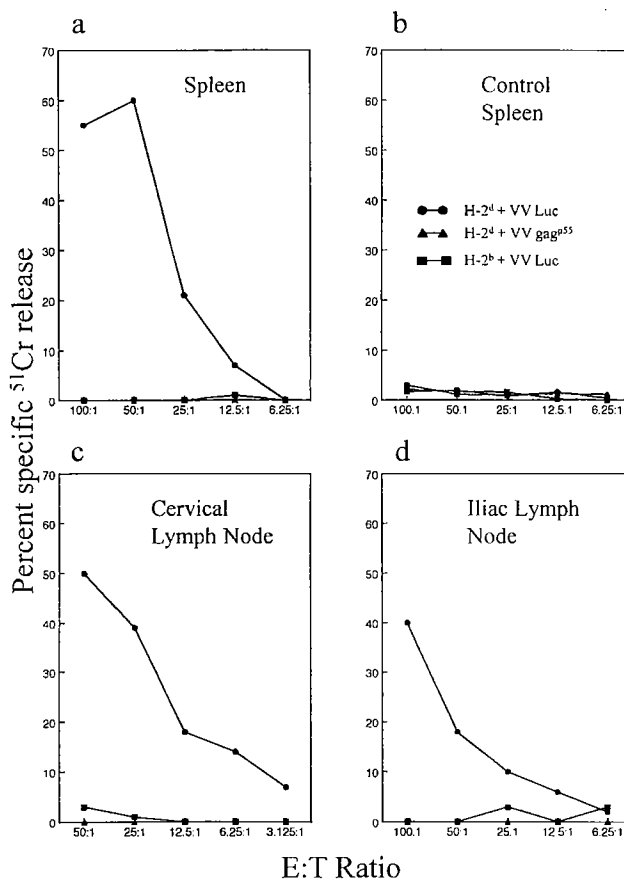
intranasal administration, we demonstrated distribution of plasmid DNA and also the encoded transcripts throughout the respiratory tract, in agreement with previous observations (48–58). Expression was also detected in the gut, spleen, and cervical and mesenteric LNs, but not in the liver, brain, or ovaries (Fig. 2). It is not inconceivable that plasmid DNA is swallowed following nasal delivery, accounting for expression in the gut. Preliminary evidence indicates that enteric delivery of plasmid DNA-cytofectin complexes also elicits mucosal immunity to the encoded Ag (C. Barnfield and L. S. Klavinskis, unpublished observations). With the exception of the liver, the distribution of plasmid DNA and encoded transcripts is redolent of the dissemination of phosholipid-based liposomes to reticuloendothelial tissues (58), although the plasmid DNA-DMRIE/DOPE complex is not a classical liposome (30). It is possible that in addition to direct transfection of the nasal epithelium (demonstrated by in situ hybridization; Fig. 1*b*), plasmid DNA-lipid complexes are endocytosed by M cells in the follicle-associated epithelium and transported intact to the underlying lamina propria. Preliminary data indicate that dendritic cells are transfected in both nasal tissue and cervical LNs (C. Barnfield and L. S. Klavinskis, unpublished observations). However, it is currently unclear whether free plasmid DNA-cytofectin complexes

drain via lymph to the regional LNs or whether dendritic cells or macrophages that are intimately associated with the basolateral surface of M cells (59) are directly transfected and migrate to the regional LNs. Conceivably, both mechanisms are operable, and this is under investigation. Alternatively, from the highly vascularized tissue of the nasopharynx, plasmid DNA complexes may directly enter the circulation and disseminate to the spleen, priming for the systemic immune responses reported herein.

The persistence of the encoded protein in nasal tissue to residual levels by day 28 and the short term expression of transcripts in the lung are consistent with previous observations addressing cytofectin-mediated gene delivery to the respiratory tract (48–51). While short term expression may be undesirable for gene replacement therapy, in the context of mucosal vaccination such short term expression may be an attribute to prevent the induction of systemic tolerance reported previously with repeated exposure of Ag (60). Whether low levels of Ag and specific transcripts are expressed below the detection of our assays is unclear.

In conclusion, this study demonstrates that intranasal immunization with plasmid DNA-lipid complexes is effective in eliciting Ag-specific CTL and sIgA in the genital and rectal tracts, with specific IgA and IgG Abs and CTL also induced in the systemic compartment. A vaccine capable of stimulating these responses may prove effective in the prevention of sexually transmitted disease, consistent with the current hypothesis that a succession of cellular and humoral immune barriers from the genital mucosa may be required (57). Studies are currently in progress to determine the efficacy of this simple, noninvasive plasmid DNA immunization strategy in preventing genito-rectal infection of macaques with SIV.

## Acknowledgments

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